

Prevalence and Sequence Variants of IS481 in *Bordetella bronchiseptica*: Implications for IS481-Based Detection of *Bordetella pertussis*[†]

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We report the prevalence in *Bordetella bronchiseptica* of IS481, a frequent target for diagnosis of *Bordetella pertussis*, as approximately 5%. However, PCR amplicons of the predicted size were detectable in 78% of IS481-negative strains. Our results suggest that PCR targeting IS481 may not be sufficiently specific for reliable identification of *B. pertussis*.

Bordetella pertussis is the etiologic agent of whooping cough, an acute respiratory disease occurring exclusively in humans. Widespread vaccination provides a high level of protection in children, but unvaccinated populations and cohorts in whom vaccination-induced immunity has waned remain at risk. Recently, a reemergence of the disease within highly vaccinated populations has occurred, and transmission from adult reservoirs to unimmunized or partially immunized children is of increasing concern in many areas of the world (36). A sensitive and specific method for identification of *B. pertussis* is crucial for monitoring prevalence, accurately defining epidemiology, and optimizing intervention and prevention strategies, including management of outbreaks. Culture remains the diagnostic standard but suffers from poor sensitivity, slow turnaround time, and practical difficulties. Additionally, it is necessary to distinguish between *B. pertussis* and *Bordetella parapertussis*, which may cause a milder pertussis-like syndrome in humans. PCR assays that differentiate between these bacteria, either alone or combined with traditional culture, are increasingly being implemented in diagnostic laboratories.

A frequently used target for PCR detection of *B. pertussis* is the repetitive element IS481 (29, 30), which is absent in *B. parapertussis* (8, 48) but found in approximately 50 to a few hundred copies in *B. pertussis* (16, 27, 34, 35, 42). While IS481 is generally regarded as specific for *B. pertussis*, few studies definitively examine the prevalence of IS481 in the closely related agent *Bordetella bronchiseptica*. Despite recent evidence that *B. pertussis* may have evolved from a human-associated lineage of *B. bronchiseptica* (8), the latter agent is primarily associated with disease in mammals other than humans and, therefore, has been considered to have little clinical significance. However, human illness and carriage associated with *B. bronchiseptica* is on the rise, particularly

in infants or immunocompromised hosts with exposure to carrier animals (1, 4, 5, 6, 11, 14, 15, 17, 26, 31, 32, 38, 44, 52, 54, 55). While rare, disease in immunocompetent adults may also occur (5, 22, 26, 54).

Analysis of a few isolates by Southern blotting suggested that IS481 may not be found in *B. bronchiseptica* (19, 27, 34), but one study provided evidence for a single copy in an isolate of unstated origin (16). van der Zee et al. (50) reported a prevalence of approximately 1% based on a diverse group of 144 strains. Unfortunately, the data upon which this conclusion is based were not presented, and specific methods used to identify IS481 are not detailed either in the original report or in the supporting references provided (49, 51). A more recent investigation evaluating 91 *B. bronchiseptica* isolates from a variety of host species identified only 2 (both equine) positive by PCR for an amplicon derived from the central region of IS481 (8). From a clinical perspective, it is important to know whether amplicons can be generated from *B. bronchiseptica* strains using IS481-specific primers designed to detect *B. pertussis*, potentially leading to an erroneous diagnosis. A review of the literature encompassing PCR methods used to identify *B. pertussis* reveals that “diagnostic” amplicons were obtained from *B. bronchiseptica* isolates in at least five investigations using primers specific for the 5′ end of the repetitive element (7, 13, 16, 20, 21) but by none using primers that target the 3′ end (3, 9, 10, 45). No diagnostic PCRs targeting the central region of IS481 have been proposed. Only about 20 *B. bronchiseptica* isolates were evaluated overall in these studies (the exact number being unclear from the details provided), and only a few were obtained from humans. The goal of the present study was to establish the prevalence of IS481 based on a larger number of *B. bronchiseptica* isolates, including those of human origin, and to assess the potential for misidentification of IS481-containing strains of *B. bronchiseptica* as *B. pertussis* when using IS481-specific PCR primers.

PCR primers IS481-1 and IS481-2 (Fig. 1) were used to screen for IS481 in *B. bronchiseptica*. This primer set is used by the Pertussis Program Laboratory of the Centers for Disease Control and Prevention for IS481-based identification of *B. pertussis* and encompasses a 252-bp region of the insertion sequence frequently selected as a target in *B. pertussis* diag-

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		1	IS481-5 →								
Bp	IS481		CTAGGTGTGA	AGATTCAATA	GGTTGTATGC	ATGGTTCATC	CGAACCGGAT	TTGAGAAACT	GGAAATCGCC	AACCCCCCAG	
Bp	IS481v1		---TC---	-----	-----	-----	-----	-----	-----	G-----	
Bp	IS481v2		---C---	-----	-----	-----	-----	-----	-----	-----	
Bb	IS481v1			-----	-----	-----	-----	-----	-----	-A-----	
Bb	IS481v2			-----	-----	-----	-----	-----	-----	-A-----	
		81	IS481-7 →							←	
Bp	IS481		TTCACCTCAAG	GAGCCCGGCC	GGATGAACAC	CCATAAGCAT	GCCCGATTGA	CCTTCCTACG	TCGACTCGAA	ATGGTCCAGC	
Bp	IS481v1		-----	-----	-----	-----	-----	-----	-----	-----	
Bp	IS481v2		-----	-----	-----	-----	-----	-----	-----	-----	
Bb	IS481v1		-----	-----	-----	-----	-----	-----	-----	-----	
Bb	IS481v2		-----	-----A-	-----	-----	-----	-----	-----	-----	
			← IS481-10		← IS481-6			← IS481-8			
Bp	IS481		AATTGATCGC	CCATCAAGTT	TGTGTGCCTG	AAGCGGCCCG	CGCCTATGGG	GTACCCGCGC	CGACTGTGCG	CAAATGGCTG	
Bp	IS481v1		-----	-----	-----	-----	-----	-----	-----	-----	
Bp	IS481v2		-----	-----	-----	-----	-----	-----	-----	-----	
Bb	IS481v1		-----	-----	-----	-----	-----	-----	-----	-----	
Bb	IS481v2		-----G--A-	-----	---C---T--	-----	-----	-----	-----	-----	
		241									
Bp	IS481		GGCCGCTTCC	TGGCTCAGGG	CCAGGCGGGC	TTGGCCGATG	CGTCCTCGCG	CCCGACGGTC	TCGCCCCGAG	CGATTGCGCC	
Bp	IS481v1		-----	-----	-----	-----	-----	-----	-----	-----	
Bp	IS481v2		-----	-----	-----	-----	-----	-----	-----	-----	
Bb	IS481v1		-----	-----	-----	-----	-----	-----	-----	-----	
Bb	IS481v2		-----	-----A--	-----	C-----C-	-----	-----	-----	-----	
		321									
Bp	IS481		GGCCAAGGCG	CTGGCTATCG	TGGAGCTGCG	CCGCAAGCGG	CTGACCCAAG	CGCGCATCGC	CCAGGCGCTG	GGCGTGTCAG	
Bp	IS481v1		-----	-----	-----	-----	-----	-----	-----	-----	
Bp	IS481v2		-----	-----	-----	-----	-----	-----	-----	-----	
Bb	IS481v1		-----	-----	-----	-----	-----	-----	-----	-----	
Bb	IS481v2		-----	-----C---	-----	-T-----	-----	-----	-----C---	-----	
		401									
Bp	IS481		CCAGCACCGT	CAGCCGCGTC	CTGGCCCGCG	CCGGTCTGTC	GCACCTGGCC	GACCTGGAGC	CGGCCGAGCC	GGTGGTGCGC	
Bp	IS481v1		-----	-----	-----	-----	-----	-----	-----	-----	
Bp	IS481v2		-----	-----	-----	-----	-----	-G-----	-----	-----	
Bb	IS481v1		-----	-----	-----	-----	-----	-----	-----	-----	
Bb	IS481v2		-----	-----	-----	-----	---T-----	-----	-----	-----	
		481									
Bp	IS481		TACGAGCATC	AGGCCCCCGG	CGATCTGCTG	CACATCGACA	TCAAGAAGCT	GGGACGTATC	CAGCGCCCTG	GCCACCGGGT	
Bp	IS481v1		-----	-----	-----	-----	-----	-----	-----	-----	
Bp	IS481v2		-----	-----	-----	-----	-----	-----	-----	-----	
Bb	IS481v1		-----	-----	-----	-----	-----	-----	-----	-----	
Bb	IS481v2		-----A---	-----	-----	-----	-----	-----	-----	-----	
		561	IS481-1 → IS481-9 →								
Bp	IS481		CACGGGCAAC	CGACGCGATA	CCGTTGAGGG	GGCCGCGTGG	GACTTCGTCT	TCGTGGCCAT	CGATGACCAC	GCCCCGCTGG	
Bp	IS481v1		-----	-----	-----	-----	-----	-----	-----	-----	
Bp	IS481v2		-----	-----	-----	-----	-----	-----	-----	-----	
Bb	IS481v1		-----	-----	-----	-----	-----	-----	-----	-----	
Bb	IS481v2		-----T---	-----	-----	-----	-----	-----	-----	-----	
		641	IS481-13 →								
Bp	IS481		CCTTCACCGA	CATCCACCCC	GACGAGCGCT	TCCCCAGCGC	CGTCCAGTTC	CTCAAGGACG	CAGTGGCCTA	CTACCAGCGC	
Bp	IS481v1		-----	-----C---	-----	-----	-----	-----	-----	-----	
Bp	IS481v2		-----	-----	-----	-----	-----	-----	-----	-----	
Bb	IS481v1		-----	-----	-----	-----	-----	-----	-----	-----	
Bb	IS481v2		-----	-----G-----	-----	-----	-----	-----	-----	-----	
		721	IS481-11 →							ORF 3	
Bp	IS481		CTGGGCGTGA	CCATCCAGCG	CTTGCTCACC	GACAATGGCT	CGGCCTTTCG	CAGCCGCGCC	TTCGCCGCGC	TGTGCCATGA	
Bp	IS481v1		-----	-----	-----	-----	-----	-----	-----	-----	
Bp	IS481v2		-----	-----	-----	-----	-----	-----	-----	-----	
Bb	IS481v1		-----	-----	-----	-----	-----	-----	-----	-----	
Bb	IS481v2		-----T---	-----	-----	-----	-----	-----	-----	-----G---	

FIG. 1 continues

FIG. 1—continued

	801	← IS481-2 ←							
Bp IS481	GCTGGGCATC	AAGCACCGCT	TTACCCGACC	TTACCGCCCA	CAGACCAATG	GCAAGGCCGA	ACGCTTCATC	CAGTCGGCCT	
Bp IS481v1	-----	-----	-----	-----	-----	-----	-----	-----	
Bp IS481v2	-----	-----	-----	-----	-----	-----	-----	-----	
Bb IS481v1	-----	-----	-----	-----	-----	-----	-----	-----	
Bb IS481v2	-----	-----	-----	-----	-----	-----	-----	-----	
	881	← IS481-16							
Bp IS481	TGCGTGAGTG	GGCTTACGCT	CACACCTACC	AGAACTCCCA	ACACCGAGCC	GATGCCATGA	AATCCTGGCT	ACACCACTAC	
Bp IS481v1	-----	-----	-----	-----	-----	-----	-----	-----	
Bp IS481v2	-----	-----	-----	-----	-----	-----	-----	-----	
Bb IS481v1	-----	-----	-----	-----	-----	-----	-----	-----	
Bb IS481v2	-----	---C---C	-----	-A-----	-----C--	-----	-----	-----	
	961	← IS481-14				← IS481-12			
Bp IS481	AACTGGCATC	GACCCACCA	AGGCATCGGG	CGCGCTGTAC	CCATCTCCAG	ACTCAACCTG	GACGAATACA	ACCTATTGAA	
Bp IS481v1	-----	-----	-----	-----	-----	-----	-----	-----C	
Bp IS481v2	-----	-----	-----	-----	-----	-----	-----	-----C	
Bb IS481v1	-----	-----	-----	-----	-----	-----	-----	-----	
Bb IS481v2	-----	-G---T----	---T-----	-----	-----	-----	-----	-----	
	1041								
Bp IS481	TCTTCACAGC	TAG							
Bp IS481v1	AG-----	--T							
Bp IS481v2	AG-----	---							
Bb IS481v1									
Bb IS481v2									

FIG. 1. Sequences and locations of IS481 primers used in this study and comparison of *B. bronchiseptica* IS481 sequence variants with those reported for *B. pertussis* IS481. The top line shows the sequence originally reported for IS481 from *B. pertussis* Tohama (Bp IS481) (27). The sequence of *B. pertussis* IS481 variant 1 (Bp IS481v1) was also derived from the Tohama strain but from a different copy of IS481 (28). The sequence of *B. pertussis* IS481 variant 2 (Bp IS481v2) was derived from the Wellcome 28 strain (28). *B. bronchiseptica* IS481 variants 1 and 2 (Bb IS481v1 and Bb IS481v2, respectively) are also shown. Dashes represent conserved bases; substitutions are indicated by the appropriate letter. Primer locations and directions are indicated by shaded areas and arrows, respectively. Bases 601 to 612 are more darkly shaded to represent a region of overlap between the 3' end of IS481-1 and the 5' end of IS481-9. The proposed start codon and direction of transcription for ORF3 (27) are specified by bold, underlined bases and an arrow, respectively. The terminal inverted repeats are underlined. Nucleotide positions are numbered according to the sequence numbering of McLafferty et al. (27).

nostic PCRs (3, 9, 10, 45, 53). Reaction mixtures included 0.4 μ M each primer, 1 U AmpliTaq polymerase (Applied Biosystems, Foster City, CA), 2.5 μ l 10 \times buffer II (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2.5 mM MgCl₂, 200 μ M deoxynucleotide triphosphates, and either 100 ng of purified DNA (n = 17), 0.5 μ l of a cell lysate (n = 9) (41), or in separate reactions, both (n = 123), in a final volume of 25 μ l. PCR was carried out in an Applied Biosystems (Foster City, CA) 9700 thermal cycler. Cycling conditions were as follows: 3 min at 95°C; 35 cycles, with 1 cycle consisting of 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s; and a final extension step of 72°C for 10 min. A total of 149 *B. bronchiseptica* isolates were evaluated (see Table S1 in the supplemental material). These were obtained primarily from humans and companion or farm animal species; some have previously been examined for IS481 by other methods (8, 50). *B. pertussis* (Tohama) was used as a positive control. The integrity of templates and reagents was established by parallel testing with a 16S rRNA-specific PCR (41). Five microliters of each PCR mixture was analyzed by agarose gel electrophoresis.

Unexpectedly, an amplicon of the predicted size was detected from 118 *B. bronchiseptica* isolates, including 22 of 24 isolates of human origin, following PCR with primer pair IS481-1/IS481-2. Strain RB50, included as one of the negative controls since it does not contain IS481 (35), gave false-positive results. One or two additional bands were present in re-

actions from 12 isolates. No amplification products were visualized from negative controls containing water in place of template, included with every set of PCRs. Representative results are shown in Fig. 2A and B. Increasing the annealing temperature to 60°C (primer melting temperature is 62 to 63°C) reduced the intensities of some weakly fluorescent amplicons but did not completely eliminate them. Significant differences in band intensity apparent in reaction mixtures with a standardized amount of purified DNA (Fig. 2A) suggest that some isolates may contain one or more copies of IS481, although it seems likely that weaker amplicons of the expected size represent false-positive results.

The strongly fluorescent amplicons of ~250 bp generated with purified DNA from three *B. bronchiseptica* isolates, representing three different species of origin, were each purified with spin columns (QIAGEN, Valencia, CA) and sequenced directly (minimum of three reactions, at least one from each strand) as described previously (39). Sequence alignment showed 98.8 to 100% identity with the sequence originally reported for this region of *B. pertussis* IS481 (27; K. B. Register, unpublished data). The specificities of the IS481-1/IS481-2 PCR products from the 118 positive isolates were evaluated by Southern blotting, as described previously (40), using as a probe one of the sequenced *B. bronchiseptica* amplicons labeled with digoxigenin. Following exposures of 30 min or less, an intense signal was evident only from the ~250 bp product of

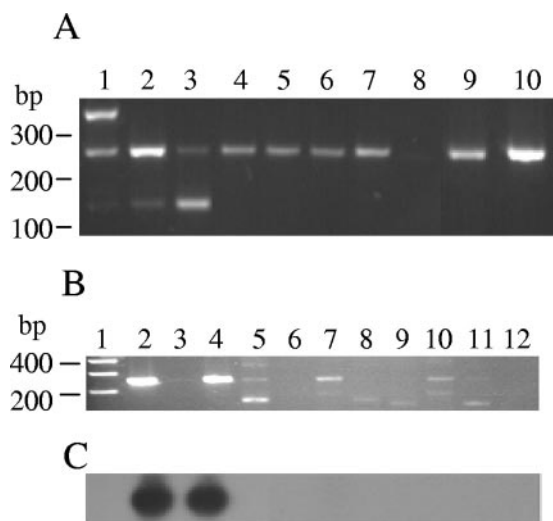


FIG. 2. Amplicons from PCRs with IS481-1/IS481-2 primers and *B. bronchiseptica* purified DNA (A) or cell lysates (B) detected by ethidium bromide staining. (A) Lanes: 1, rabbit isolate (M116919/02/1); 2, dog isolate (MBORD595); 3, guinea pig isolate (MBORD673); 4, cat isolate (MBORD733); 5, human isolate (W48661); 6, turkey isolate (4448); 7, rabbit isolate (RB50); 8, negative control; 9, dog isolate (MBORD599); 10, *B. pertussis*. Relative positions of DNA size markers are indicated to the left of the gel. (B) Lanes: 1, DNA size markers; 2, *B. pertussis*; 3 and 4, guinea pig isolates (MBORD762 and MBORD665, respectively); 5, dog isolate (MBORD839); 6, pig isolate (5238); 7, human isolate (MO289); 8, rabbit isolate (MBORD835); 9, pig isolate (SG8); 10 and 11, rabbit isolates (RB50 and M602871/00/1, respectively); 12, negative control. (C) Southern blot of the gel in panel B hybridized with an IS481-specific probe as described in the text.

seven isolates (Table 1; see Table S1 in the supplemental material), all of which displayed strongly fluorescent amplicons of this size in ethidium bromide-stained gels. Representative results are shown in Fig. 2C.

TABLE 1. Prevalence and host species distribution of IS481 in the *B. bronchiseptica* isolates included in this study

Host species	No. of isolates	
	IS481 positive ^a	IS481 negative ^b
Human	0	2 (22) ^c
Dog	1	1 (28)
Cat	0	0 (8)
Horse	1	0 (6)
Rabbit	0	3 (17)
Guinea pig	5	1 (5)
Pig	0	10 (17)
Turkey	0	2 (11)
Seal	0	3 (2)
Sea otter	0	1 (0)
Leopard	0	0 (1)
Unknown	0	1 (1)
Total	7	24 (118)

^a Numbers of isolates in which an amplicon of the predicted size could be detected in agarose gels and which hybridized with an IS481-specific probe.

^b Numbers of isolates from which no amplicon could be detected in agarose gels.

^c The numbers in parentheses are the numbers of isolates for which an amplicon of the predicted size that failed to hybridize with an IS481-specific probe was present (false positives).

The 7 confirmed IS481-positive isolates, as well as 48 PCR-positive isolates whose amplicons failed to hybridize with the IS481 probe, were further evaluated by Southern blotting with genomic DNA. Following digestion of 3 μ g of purified DNA with PstI, for which no recognition sites are present in *B. pertussis* IS481 (27, 28), fragments were separated on 0.6% agarose gels, and blots were prepared and hybridized with the *B. bronchiseptica* IS481 probe as described above. Signals were detected only from the seven isolates already identified as containing IS481, and at least some appeared to contain more than a single copy (K. B. Register, unpublished data). Additional Southern blot analysis using alternative restriction enzymes clearly demonstrated all seven isolates possess multiple copies of IS481. Results obtained with NarI, which has the most frequently occurring recognition sequence of the enzymes tested, are shown in Fig. 3.

These data suggest that moderately to weakly fluorescent IS481-1/IS481-2 amplicons, although of the predicted size, are not derived from IS481. Amplicons obtained from six IS481-negative *B. bronchiseptica* isolates, including RB50, were sequenced in an attempt to determine their origin. All sequences shared 100% identity with one another as well as with the 5' and flanking region of an RB50 open reading frame predicted to encode an extracellular solute-binding protein (locus tag BB5004), identified from a BLASTN search of the genome database (http://www.sanger.ac.uk/Projects/B_bronchiseptica). Alignment of the IS481 primers with this segment of the RB50 genome revealed likely primer-binding locations which would result in a product of 252 bp (Fig. 4), consistent with our observations. No other regions of significant identity with the IS481-1/IS481-2 amplicon were noted within the sequence of

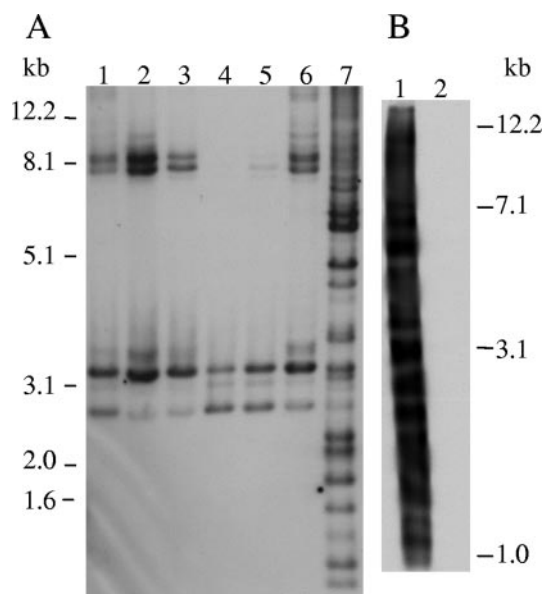


FIG. 3. Southern blots with NarI-digested *B. bronchiseptica* genomic DNA or, for comparison, *B. pertussis* genomic DNA hybridized to an IS481-specific probe. (A) Lanes: 1, dog isolate (MBORD595); 2 to 6, guinea pig isolates (MBORD665, MBORD666, MBORD668, MBORD669, and MBORD678, respectively); 7, horse isolate (MBORD731). (B) Lanes: 1, *B. pertussis*; 2, *B. bronchiseptica* RB50. Relative positions of DNA size markers are indicated to the left (A) or right (B) of the gel.

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                    5326853                                5327104
                    |                                      |
BB 5004    ...ATCGAACTGGGCTCCGTCTTCAACGCCACC...CTTACCTTTACGCAGATGCTTGCCATGTCCTCTGTA...
IS481-1    GCCGGCTGGGACTTCGTCTTC
rcIS481-2    CTTTACCC GA CCTTACC G CCCACAG

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FIG. 4. Potential binding sites for primers IS481-1 and IS481-2 (shown as the reverse complement [rcIS481-2]) within and flanking the BB5004 locus of the RB50 genome. Base matches are indicated in bold type. Coordinates shown are from the genome database sequence. The RB50 sequence in the intervening segment is 100% identical to the sequence derived from false-positive amplicons, as described in the text.

the extracellular solute-binding protein gene spanned by the primers.

Because of this unexpected finding, additional analysis was carried out to assess the potential for unintended primer binding to DNA from other genera that might contaminate clinical samples or be inadvertently introduced from the environment. A BLAST search of the GenBank RefSeq database revealed regions of 100% identity with the 13 to 15 consecutive 3'-terminal bases of IS481-1 in sequences derived from *Aspergillus oryzae*, *Candida albicans*, and *Streptomyces avermitilis* and of IS481-2 in sequences from *Saccharomyces cerevisiae*, *Homo sapiens*, and multiple strains of both *Streptococcus pyogenes* and *Escherichia coli*. However, when the database was queried with a single sequence comprised of the concatenated primers and an N₂₂ spacer, no alignments with the potential to result in amplification of a product were identified. These results suggest that while there may be sequences in human DNA and some bacterial and fungal species capable of weakly binding IS481-1 or IS481-2, a PCR amplicon is not likely to be generated.

Since some *B. bronchiseptica* strains possess at least a portion of IS481, misidentification of such strains as *B. pertussis* could occur when IS481-based PCR assays are used as the basis for a diagnosis of whooping cough. To more precisely evaluate the potential for misidentification with particular primer sets, we determined the DNA sequence of the *B. bronchiseptica* insertion element. Primers specific for the 5' and 3' ends of *B. pertussis* Tohama IS481 (IS481-5 and IS481-12 [Fig. 1]) were used to generate amplicons comprising the entire insertion element from the seven IS481-containing *B. bronchiseptica* isolates. Amplicons were purified and directly sequenced as described above. Sequence alignment revealed two variants of IS481, neither of which is identical to any of the three variants reported for *B. pertussis* (Fig. 1). It should be noted that, since all positive isolates have multiple copies of IS481 and the DNA sequences were obtained from purified PCR products, it is not possible to rule out the existence of additional, less frequently occurring variants. *B. bronchiseptica* IS481 variant 1 was found only in isolate MBORD731 and shares the greatest degree of identity with *B. pertussis* variants (one to three base substitutions depending on the variant used for comparison). The IS481 sequences from the six remaining *B. bronchiseptica* isolates are identical to one another (*B. bronchiseptica* IS481 variant 2) and have 25 to 27 base substitutions compared to the *B. pertussis* variants. No base substitutions are shared between *B. pertussis* IS481 variants and those of *B. bronchiseptica*. Interestingly, one substitution in *B. bronchiseptica* IS481 variant 2 eliminates a purported start codon for ORF3 (28), suggesting the insertion sequence may not be functional in most IS481-containing *B. bronchiseptica* isolates

and perhaps explaining both its low prevalence and copy number. The single *B. bronchiseptica* isolate with an intact ORF3 start codon contains many more copies of IS481 than the other positive isolates. Alternatively, if IS481 had been acquired relatively recently, insufficient time may have elapsed for generating additional copies.

On the basis of a comparison of the *B. bronchiseptica* IS481 sequences with those of IS481 primers used for conventional PCR identification of *B. pertussis* (2, 3, 9, 10, 12, 13, 16, 18, 20, 21, 23, 25, 37, 45, 46, 47, 53), it seems likely that misidentification of IS481-containing strains of *B. bronchiseptica* would occur. Many of the suggested primers are 100% identical to the *B. bronchiseptica* sequence reported here, while others have a few base substitutions which may still permit amplification. A subset of the most commonly used primer pairs, including IS481-5/IS481-6 (18, 23, 46, 47), IS481-7/IS481-8 (21), IS481-5/IS481-8 (25), IS481-5/IS481-10 (13, 16), IS481-9/IS481-12 (9), IS481-11/IS481-14 (53), and IS481-13/IS481-16 (45; see Fig. 1 for primer sequences and locations), was tested with genomic DNA from the seven *B. bronchiseptica* isolates containing IS481. Selection of an appropriate melting temperature during cycling was problematic, since the temperature used by different investigators for the same primer pair sometimes varied greatly (e.g., a range of 55°C to 66°C for IS481-5/IS481-6) and many primer pairs are poorly matched (some differing by 10°C or more). In keeping with the conditions reported most frequently, primer pairs were tested at both 55°C and 58°C. PCR components and other cycling parameters were as described above. Amplicons were obtained from all isolates at both melting temperatures with all primer pairs tested, including those having one or more mismatched bases in comparison with *B. bronchiseptica* IS481 sequence. Representative results are shown in Fig. 5. Although no false-positive results were noted with strain RB50, faint bands ~100 to 150 bp larger and/or smaller than the IS481-specific amplicons were evident for a few of the *B. bronchiseptica* isolates using two of the three primer pairs directed against the 3' portion of the insertion element (K. B. Register, unpublished data).

Our data indicate an IS481 prevalence of roughly 5% in the group of *B. bronchiseptica* isolates examined (Table 1); others have reported a prevalence of roughly 1 to 2% (8, 50). The isolates evaluated by Diavatopoulos et al. (8) include 35 also evaluated here. Our results are concordant with the exception of strain MBORD669, previously reported to be IS481 negative but found here to be positive. Comparison of the sequence of MBORD669 IS481 with the primers used by Diavatopoulos et al. reveals the sequences are identical except for a single, internal base mismatch in the reverse primer, which seems unlikely to explain the discrepancy. Accidental cross-contamination of cultures in our laboratory also seems unlikely since

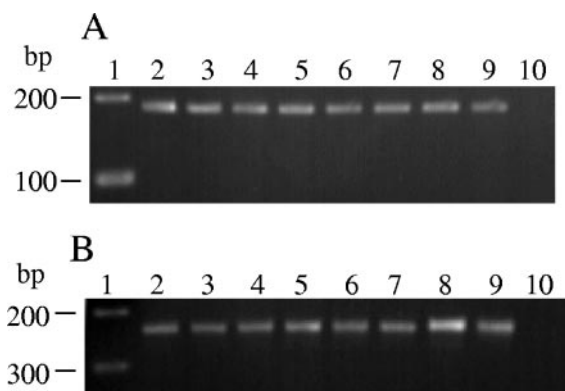


FIG. 5. Amplicons obtained from PCR with primers targeting the 5' end (IS481-5/IS481-6 [A]) or 3' end (IS481-13/IS481-16 [B]) of *B. pertussis* IS481 using *B. bronchiseptica* genomic DNA and a melting temperature of 58°C. Lanes: 1, DNA size markers; 2, MBORD595; 3, MBORD665; 4, MBORD666; 5, MBORD668; 6, MBORD669; 7, MBORD678; 8, MBORD731; 9, *B. pertussis*; 10, RB50. Relative positions of DNA size markers are indicated to the left of the gels.

the PvuII ribotype of MBORD669 is unique among our IS481-positive isolates (40; K. B. Register, unpublished data). A comparative evaluation of MBORD669 DNA obtained from each laboratory could perhaps assist in resolving the discrepancy. The remaining study (50), in which the method used to identify IS481 was not detailed, included 76 strains examined here. Our results are in agreement with theirs, except for five isolates reported here as positive for IS481 which were previously reported as IS481 negative. In the absence of additional experimental details, the reason for this discrepancy is unclear. It could be postulated that our results are confounded by the presence of IS1002, an insertion sequence closely related to IS481 found in multiple *Bordetella* species including, rarely, *B. bronchiseptica* (8, 49). However, the degree of sequence identity between IS1002 (49) and our IS481 probe is insufficient to withstand the stringency of the conditions used for hybridizations. Detection of many identically sized restriction fragments with the IS481 probe in all strains positive by Southern blotting (Fig. 3A; K. B. Register, unpublished data) also argues against cross-hybridization of the probe with IS1002.

The prevalence and sequences of IS481 in *B. bronchiseptica* reported here suggest that the specificity of this target for identification of *B. pertussis* is less than presently perceived. Although the insertion sequence was not detected in any of the 26 human isolates tested, its occurrence in strains obtained from farm and companion animals suggests that transmission of IS481-containing *B. bronchiseptica* strains to humans remains a possibility. Also of concern is the finding that secondary amplicons not derived from IS481 but of the predicted size, as well as some with slightly different mobilities, were often apparent when using some primers derived from the 3' region of *B. pertussis* IS481. Utilization of amplicon-specific probes in real-time PCR formats may alleviate this problem, but careful evaluation of IS481-based diagnostic assays for *B. pertussis* is essential, including an analysis of proven IS481-positive and -negative strains of *B. bronchiseptica*. It is unclear whether *B. bronchiseptica* isolates reported as PCR positive in assays targeting the 5' region of IS481 are, in fact, IS481 positive (7,

13, 16, 20, 21). The details available suggest that at least some amplicons may have been derived from secondary primer-binding sites (16, 21) as observed for most isolates in this study with primers IS481-1/IS481-2.

Our observations, together with reports identifying numerous IS481-positive *Bordetella holmesii* strains (21, 24, 42, 45, 46, 47), raise significant concerns as to the likelihood of a false diagnosis of *B. pertussis* if IS481 PCR is the only test carried out, as observed in a recent quality assessment proficiency panel (33). All laboratories utilizing IS481 PCR mistakenly identified both *B. bronchiseptica* and *B. holmesii* as *B. pertussis*. The suggestion that IS481 PCR assays are sufficiently specific for diagnosis of *B. pertussis* (43) may be premature. Consequently, promising alternative and/or confirmatory PCR targets should continue to be evaluated, and clinical sensitivity and specificity of IS481-based PCR for pertussis need to be more rigorously defined.

Nucleotide sequence accession numbers. The GenBank accession numbers for the DNA sequences determined in this study are EF043395 to EF043401.

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